Individual and synergistic effects of sniffing frequency and flow rate on olfactory bulb activity

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Individual and synergistic effects of sniffing frequency and flow rate on olfactory bulb activity, *J Neurophysiol* 106: 2813–2824, 2011. First published September 7, 2011; doi:10.1152/jn.00672.2011.—Is faster or stronger sniffing important for the olfactory system? Odorant molecules are captured by sniffing. The features of sniffing constrain both the temporality and intensity of the input to the olfactory structures. In this context, it is clear that variations in both the sniff frequency and flow rate have a major impact on the activation of olfactory structures. However, the question of how frequency and flow rate individually or synergistically impact bulbar output has not been answered. We have addressed this question using multiple experimental approaches. In double-tracheotomized, anesthetized rats, we recorded both the bulbar local field potential (LFP) and mitral/tufted cells’ activities when the sampling flow rate and frequency were controlled independently. We found that a tradeoff between the sampling frequency and the flow rate could maintain olfactory bulb sampling-related rhythmicity and that only an increase in flow rate could induce a faster, odor-evoked response. LFP and sniffing were recorded in awake rats. We found that sampling-related rhythmicity was maintained during high-frequency sniffing. Furthermore, we observed that the covariation between the frequency and flow rate, which was necessary for the tradeoff seen in the anesthetized preparations, also occurred in awake animals. Our study shows that the sampling frequency and flow rate can act either independently or synergistically on bulbar output to shape the neuronal message. The system likely takes advantage of this flexibility to adapt sniffing strategies to animal behavior. Our study provides additional support for the idea that sniffing and olfaction function in an integrated manner.

JUST AS VISUAL PERCEPTION is dependent on eye movement, olfaction is dependent on the way that odors are sampled (i.e., respiration). Olfactory activity and odor-sampling behaviors maintain strong temporal relationships at multiple levels, including the olfactory receptors (Carey et al. 2009; Chaput 2000), glomerular activation maps (Spors and Grinvald 2002), mitral/tufted cells (Chaput et al. 1992; Macrides and Chorover 1972; Margrie and Schaefer 2003; Onoda and Mori 1980; Sobel and Tank 1993), bulbar local field potential (LFP) (Adrian 1942; Buonviso et al. 2003), and the piriform cortex (Litaudon et al. 2003; Poo and Isaacson 2009; Wilson 1998). Odor sampling behavior is thus a key feature of olfactory perception (Mainland and Sobel 2006), and sniffing has been proposed to be an olfactory motor act (Johnson et al. 2003). If this is indeed the case, any variation in the sniffing frequency and/or flow rate should shape olfactory coding. Sniffing has been shown to be highly variable with respect to both the frequency (which ranges from 2 to 12 Hz) and the flow rate (Youngentob et al. 1987). Flow rate has been observed to impact both the olfactory epithelium (Kent et al. 1996; Mozell 1970; Scott-Johnson et al. 2000) and glomerular activity (Oka et al. 2009). Recently, we described (Courtiol et al. 2011) flow rate-induced modifications of bulbar activity. How variation in the sampling frequency impacts bulbar activity remains unclear. At least two factors could be modified by a high frequency. The first factor is the strength of the bulbar response, because a high sampling frequency results in a short bulbar activation period, as confirmed by the attenuation of the respiratory pattern of glomerular activation (Verhagen et al. 2007). The second factor that is likely modified by a high frequency is the latency of the bulbar response, because the high frequency increases the speed of odorant acquisition (Wesson et al. 2009). While the present study was under review, the effect of sampling frequency on M/T cell activity was described (Carey and Wachowiak 2011). However, the question of how the frequency and flow rate individually or synergistically impact bulbar output has not yet been addressed.

Our question was then two-fold: does a high sampling frequency modify the strength and/or latency of the bulbar response? If so, can a higher flow rate counterbalance the effects of a high frequency? To precisely control both of the sampling parameters, we used an anesthetized, double-cannulated, tracheotomized rat experimental preparation. This preparation allowed flow rate and frequency to be independently controlled, which is not possible in awake animals. We found that a tradeoff between the sampling frequency and the flow rate resulted in the persistence of sampling-related activity in the olfactory bulb (OB), and only an increase in the flow rate could induce M/T cells to respond earlier. With the use of awake rats, we demonstrated that a similar covariation between the frequency and flow rate, which was necessary for the tradeoff observed in the anesthetized preparation, occurred in the behaving animal.

MATERIALS AND METHODS

Experiment 1: OB Activity Recording and Control of Sampling Parameters in Anesthetized Rats

Preparation and recording. Ten male Wistar rats (200–450 g), obtained from Janvier (Le Genest-Saint-Ise, France), were anesthe-
tized with urethane (1.5 g/kg ip, with additional supplements as needed) and placed in a stereotaxic apparatus. Anesthesia was maintained by supplemental doses when necessary. LFP oscillations were used to monitor anesthesia depth. The animals were placed on a heating pad to maintain constant body temperature. All surgical procedures were conducted in strict accordance with the European Community Council directive of November 24, 1986 (86/609/EEC), and the guidelines of the French Ethical Committee and French Legislation, and surgical procedures received approval from the Lyon 1 University Ethics Committee (Direction of Veterinary Service #69387473).

TRACHEOTOMY. When all pain reflexes were abolished, a tracheotomy was performed by inserting the first cannula into the trachea, which allowed the rat to breathe freely. Next, a second cannula was inserted rostrally through the larynx into the postnasal cavity to allow air to be pushed and pulled through the nasal cavity.

ELECTROPHYSIOLOGICAL RECORDINGS. The dorsal region of the OB was exposed. Bulbar activity was recorded as a broadband signal (0.1–5 kHz) using 16-channel silicon probes (NeuroNexus Technologies, Ann Arbor, MI) and a homemade 16-channel direct current amplifier (gain 1,000×). Silicon probes (16-channel) were placed so that we could record both the M/T cell activity from the mitral cell layer and the maximum LFP amplitude in the granular cell layer. The mitral cell layer was located using the following criteria: LFP waveform, the magnitude of the unit action potentials, and the inability to record spikes from the granular cell layer. The granular cell layer was located by LFP waveform, as described by Buonviso et al. (2003). Recordings were performed in the whole anteroposterior axis of the OB. Data were digitally sampled at 20 kHz and acquired with a personal computer (PC) using a National Instruments (Austin, TX) acquisition card (BNC-2111).

ODORS. Odors (Sigma-Aldrich, St Louis, MO; Fluka, Germany) were delivered in a randomized series through a dilution olfactometer (400 ml/min). The odors were 2-heptanone (K07) and isoamyl acetate (ISO). The odors were delivered in front of the animal’s nose at the proportion of 18% of the saturated vapor pressure. The time delay between each odor presentation was at least 1 min. The recording protocol was as follows: 5 s of spontaneous activity, 5 s of odor-evoked activity, and 5 s of poststimulus activity.

IMPROVED NASAL AIRFLOW. Airflow was measured using fast response-time airflow sensors. This setup has been described extensively by Roux et al. (2006). We used two sensors; the first, which was placed in front of the tracheal cannula, measured the animal’s own respiration. The second sensor, which was placed at the entrance of the nostril, measured the airflow circulating through the nasal cavity. We thus recorded two airflow signals: the animal’s own respiratory airflow and the imposed nasal airflow.

To simulate respiratory cycles, we used a homemade apparatus that allowed the reproduction of both the inhalation and exhalation phases (for more details, see Courtiol et al. 2011). Briefly, the animal’s respiratory signal (collected at the tracheal cannula) was sent to the respiratory signal simulator, which in turn, sent a simulated sampling airflow toward the nasal cavity through the nasal cannula. We thus recorded two airflow signals: the animal’s own respiratory airflow and the imposed nasal airflow.

In addition to the respiratory signal, we also imposed a flow rate cycle with the basal frequency reproduced the bulb LFP signal in response to ISO, which was usually recorded in the anesthetized, nontracheotomized condition (Buonviso et al. 2003). The flow rates and sampling frequencies used corresponded to rat physiological parameters (Youngentob et al. 1987).

### Data processing

All data processing was performed using OpenElectrophy open-access homemade software (Garcia and Fourcaud-Trocme 2009). OpenElectrophy is an open source and is freely available for download at http://neuralensemble.org/trac/OpenElectrophy.

RESPIRATORY SIGNAL. An important feature of the olfactory signal is its temporal correlation with breathing. Therefore, we developed a method for representing the data as a function of the respiratory phase (Roux et al. 2006). Briefly, the respiratory cycle was first divided into two periods: inspiration and expiration. The time component for these periods was then converted into a circular-phase component, defined between zero and one, where zero and one represent the beginning of inspiration and the end of expiration, respectively. This phase representation of the respiratory cycle was used as a normalized time basis (between zero and one) and permitted us to collect and analyze the results in a standardized data format across different recordings. The respiratory-phase computation was performed on both the animal’s own respiration and the imposed nasal airflow (i.e., sampling cycles), which allowed us to compare the olfactory signal relative to either of these two signals.

- **LFPs:** LFPs were obtained by band-passing the signal at 0–200 Hz.
- **Wavelet transform and wavelet ridge extraction for γ-oscillations**
  - To preserve both time and frequency information, we used a time-frequency representation (TFR) based on a continuous wavelet transform. We have developed an algorithmic procedure (Roux et al. 2007) to extract phase information from the oscillations identified in the signal. γ-Oscillations were easily and clearly discriminated in both the TFRs and the raw signals. A γ-burst was defined as a succession of at least three oscillation cycles. An absolute threshold was defined during the stimulus epoch for the 35- to 90-Hz band and for each electrode. This threshold was used to define the time and frequency boxes centered on the points of the maximum signal amplitude. Next, the time and frequency coordinates of all local maxima in the γ-band above this threshold were extracted. From each maximum, we computed the Morlet’s complex wavelet transformation with high time resolution, both forward and backward, following the line of maximum energy. The computation stopped when the energy fell below the threshold. Therefore, for each maximum detected on the time-frequency map, we obtained, with high time resolution, a wavelet ridge, which was defined by its starting and ending times, instantaneous frequency, and instantaneous phase. Thus each γ-episode was characterized by the coordinates of its maximum (power and frequency) and its wavelet ridge (duration and frequency).

Statistical tests were performed using Excel and StatView software. The level of significance was set at \( P < 0.05 \) for all statistical tests. The occurrence of γ-oscillations (defined as the probability of observing at least one γ-burst during odorant stimulation, irrespective of the number of γ-bursts) was calculated and compared between the sampling conditions using the \( \chi^2 \) test. The average γ-burst duration, frequency, amplitude, and recurrence over successive sampling cycles (defined as the total number of oscillatory bursts/total number of sampling cycles) were calculated. These parameters were compared among the sampling conditions using two-way factorial ANOVA with the frequency and flow rate as factors. Post hoc analyses were performed using Student-Newman-Keuls test.

- **LFP sampling-related modulation**
  - In addition to γ-oscillations, LFPs presents a slower component linked to the respiratory sampling rate (Buonviso et al. 2003). LFP sampling-related modulation occurrence was determined using fast Fourier transformations (FFTs), which are better suited for processing long signals. FFTs were performed during spontaneous and odor-evoked activities. The LFP sampling-related modulation was analyzed in two different ways. First, the amplitude of the sampling-related modulation was determined. LFP signals were averaged relative to the imposed sampling cycle. Then, the amplitude of the LFP sampling-related modulation was calculated as the difference between the maxima and the minima of each averaged LFP signal. Second, the cross-correlation coefficient between the LFP signal and the imposed
nasal airflow was measured. The cross-correlations between the imposed sampling nasal airflow and the LFP signals were determined according to the following equation

\[
\frac{(X - \langle x \rangle)(Y - \langle y \rangle)}{\sigma_x \sigma_y}
\]

where \(x\) denotes the imposed sampling cycle, \(y\) is the LFP signal, \(\langle \rangle\) is the mean, and \(\sigma\) is the SD. Repeated-measures ANOVA, with the frequency and flow rate as factors, was used for comparisons of the LFP sampling-related modulation amplitude and the cross-correlation coefficients. Post hoc analyses were performed using the Student-Newman-Keuls test.

SPIKES.

- Spike sorting
  Spiking activity was extracted from individual electrodes using a band-pass filter (300–3,000 Hz). Multiunit activity consisted of a few
neurons on each electrode. We chose to use only well-discriminated units with a signal/noise ratio ≥5/1 and to sort cells according to their spike amplitude. Consequently, the number of units retained for analysis was restricted to 1–3 units/channel. We preferred to use a conservative criterion, which resulted in a limited number of units but was also highly reliable. This procedure has been described and illustrated in detail by Cenier et al. (2009).

• Pattern classification
In our preceding reports, the temporal patterns of M/T cell activity were classified according to the variations in the discharge rate with respect to the respiratory cycle (Buonviso et al. 1992; Cenier et al. 2009; Courtiol et al. 2011). Here, the imposed nasal airflow cycle was used as a time reference for pattern classification. Thus a pattern was classified as SYNCHRO when its spiking discharge presented synchronized activity relative to the imposed sampling cycle, as nonsynchronized pattern (NS) when it presented a uniform distribution of activity along the imposed sampling cycle, and as NULL when it exhibited only a few or no spikes. A M/T cell was considered responsive when the frequency rate of/and activity pattern changed from spontaneous activity to odor-evoked activity. Frequency change was considered as a response when mean and/or maximum frequency values changed by ≥2 SD between two conditions (Buonviso and Chaput 1990; Chaput et al. 1992).

• Measurements and statistics
Statistical tests were performed using Excel and StatView software. The level of significance was set at P < 0.05 for all statistical tests.
First, the percentages of odor-responsive M/T cells were calculated for each condition and compared using a χ² test. Second, the relative proportions of the different M/T cell patterns (SYNCHRO, NS, NULL) were compared among the sampling conditions during odor-evoked activities using a χ² test. Third, the latencies between the first spike and the first inspiration following odor onset were calculated and compared among the different sampling frequency conditions using two-way factorial ANOVA with the frequency and flow rate as factors. Post hoc analyses were performed using the Student-Newman-Keuls test.

Experiment 2: Respiration Recordings in Freely Moving Rats

Preparation and recording. Six male Long Evans rats (Janvier), weighing 250–300 g at the start of the experiment, were used. Food and water were available ad libitum during the experiment.

Respiration. Respiratory behavior in freely moving rats was measured using whole-body plethysmography (emka Technologies, France) with the goal of disturbing rat behavior as little as possible. This setup has been described extensively by Hegoburu et al. (2011). Briefly, the plethysmograph is composed of two chambers: a subject chamber and a reference chamber. A differential pressure transducer (Model dpt, emka Technologies) connected to both chambers allows the measurement of pressure differences. This signal reflects the respiratory activity of the rat. The measured signal was amplified, digitally sampled at 1 kHz, and acquired with a PC using an acquisition card (MC-1608FS, Measurement Computing, Norton, MA).

Protocol. It has been shown that animals increase their sniffing frequency during odor presentation (Wesson et al. 2008a), and they present a slow sniffing frequency at rest. For each rat, the respiration was recorded during 13 trials. KO7 was presented 10 times for 20 s with an intertrial interval of 4 min. With the use of this protocol, we were able to record a large range of sniffing frequencies.

Data processing. All data processing was performed using Open-Electrophy homemade open-access software (Garcia and Fourcaud-Trocmé 2009). All signals and epochs (i.e., with or without odor) were stored in a Structured Query Language database. Measurements were performed as described in detail by Hegoburu et al. (2011).

Respiratory signal. Open-Electrophy can automatically detect zero crossings of the respiratory signal, which correspond to the point of null airflow in the rising phase (Roux et al. 2006). The negative and positive phases corresponded to inspiration and expiration, respectively (see Fig. 5B). With the use of these phases, we had direct access to the frequency, inspiration time, expiration time, inspiration peak flow rate, and expiration peak flow rate. The peak flow rates and times were used to calculate the inspiration and expiration volumes, respectively.

Results
Two experimental preparations were used: a freely moving animal preparation and a double-tracheotomized, anesthetized animal preparation. We use the term “sniffing” to refer to the respiratory behavior recorded in freely moving animals, and “imposed sampling” refers to the imposed nasal airflow in anesthetized animals.

Experiment 1: Effects of Sampling Frequency and Flow-rate Variations on Bulbar Activity

Our aim in Experiment 1 was to answer two questions: does a high sampling frequency modify the strength and/or latency of the bulbar response? If so, can a higher flow rate counter-balance the effects of high frequency?
In Experiment 1, 10 anesthetized rats were used. As shown in Fig. 1A, OB activity was recorded under six different imposed sampling frequencies: 1 Hz, basal (the animal’s own respiratory frequency under urethane anesthesia; mean = 2.3 Hz ± 0.02), 4 Hz, 6 Hz, 8 Hz, and 10 Hz. These frequency conditions were coupled with two flow-rate conditions: 500 ml/min and 1,000 ml/min (Fig. 1A). When possible, two odors were used: KO7 and ISO. The 243 trials recorded from 10 rats were included in the analyses.

Effects of sampling frequency and flow rate on the strength of the bulbar response: flow rate partially counterbalances the decrease in response strength caused by reduced inspiratory duration. The strength of the bulbar response was measured via three different signals in the anesthetized rats: LFP sampling-related modulation, LFP γ-oscillations, and unitary M/T cell activity.

LFP SAMPLING-RELATED MODULATION IN THE ANESTHETIZED PREPARATION. During spontaneous activity, the occurrence of LFP sampling-related modulation decreased significantly when the sampling frequency increased, whereas increased flow rate globally enhanced LFP occurrence (data not shown). During odor presentation, LFP sampling-related modulation was observed universally, with a markedly higher power in the sampling-related frequency band in the FFT. We analyzed the amplitude of LFP sampling-related modulation during odor presentation as a function of the imposed sampling frequency (Fig. 1). As the sampling frequency increased (Fig. 1, B and D), the amplitude of LFP sampling-related modulation decreased significantly [repeated-measures ANOVA, n = 228, F (5,90) = 21.155, P < 0.0001]. This decrease was probably the result of a decrease in the sampling volume (Fig. 1A) rather than the result of a possible uncoupling between bulbar activity and imposed sampling at high sampling frequencies. Indeed, as shown in Fig. 1C, regardless of the frequency, the cross-correlation coefficients did not vary significantly as a function of frequency, except in the 1-Hz condition [Fig. 1C; repeated-measures ANOVA, n = 228, F (5,90) = 5.292, P < 0.001; post hoc Student-Newman-Keuls test, P < 0.05]. In contrast, an increase in flow rate resulted in an increase in the amplitude of LFP sampling-related modulation [repeated-measures ANOVA, n = 228, F (1,118) = 12.861, P < 0.01; Fig. 1B]. Indeed, a post hoc test revealed a significant effect (P < 0.05) at the basal, 4 Hz, 8 Hz, and 10 Hz frequencies and a similar tendency at 6 Hz (P = 0.087). An increased flow rate slightly improved the cross-correlation coefficient [repeated measures ANOVA, n = 228, F (1,118) = 6.703, P < 0.05; Fig. 1C]

With the use of these data, we were able to compare the amplitude of LFP sampling-related modulation between two conditions; for example, we compared the LFP amplitude between the basal/500-ml/min condition and the 6-Hz/1,000-ml/min condition. Interestingly, the amplitude of LFP sampling-related modulation was similar in both conditions (t-test, P > 0.05). The same observation was obtained for the 4-Hz/500-ml/min and 8-Hz/1,000-ml/min conditions, which shows that a high flow rate can compensate for the effects of high sampling frequency.

Finally, LFP sampling-related modulation still persisted at high sampling frequencies and was at least partially enhanced by an increased flow rate.

LFP γ-OSCILLATIONS IN THE ANESTHETIZED PREPARATION. With the use of our double-cannulated anesthetized preparation, we next assessed the effects of imposed sampling variations on LFP γ-oscillations. We first looked at the γ-occurrence during the entire period of odorant stimulation (the γ-occurrence was defined as the probability of observing a γ-burst, irrespective of the number of oscillatory bursts occurring during odorant stimulation). The γ-oscillation occurrence did not seem to be strongly affected by variations in the sampling frequency. Indeed, as shown in Fig. 2A, we only observed a slight decrease in the γ-oscillation in response to increased sampling frequency, and the only significant difference was between 1 Hz (occurrence = 68%) and 10 Hz [35%, χ² (1) = 4.356, P < 0.05]. In contrast, the occurrence of γ-oscillations was somewhat increased by an increased flow rate [χ² (1) = 4.31, P < 0.05]. There was no significant difference between the basal/500-ml/min and the 6-Hz/1,000-ml/min combinations or between the 4-Hz/500-ml/min and 8-Hz/1,000-ml/min combinations (χ² test, P > 0.05). Thus increased flow rate partly counterbalances the effect of high frequency, and this effect was most apparent at the highest sampling frequency. Although the global γ-occurrence was only slightly modified by the sampling frequency, we observed a change in the probability of γ-burst recurrence at each sampling cycle. Indeed, as Fig. 2, B1 and B2, shows, the γ-recurrence decreased significantly when the sampling frequency increased [ANOVA, F (5,137) = 27.505, P < 0.0001]. Notably, the γ-recurrence was higher at 1 Hz than at all other frequencies and higher at the basal frequency than at 6 Hz, 8 Hz, or 10 Hz (post hoc Student-Newman-Keuls test, P < 0.05). This decrease in the γ-recurrence was not significantly compensated for an increased flow rate [ANOVA, F (1,137) = 0.323, P = 0.57]. Indeed, a comparison of the basal/500-ml/min with the 6-Hz/1,000-ml/min combination or the 4-Hz/500-ml/min with the 8-Hz/1,000-ml/min combination revealed that the γ-recurrence was significantly higher at the low sampling frequencies and was not counterbalanced by increased flow rate.

The decrease in γ-occurrence or recurrence was not caused by a detection bias, which could have been introduced by a decrease in the amplitude of γ-oscillations. Indeed, intrinsic γ-characteristics were affected little or not at all by the frequency and flow rate; neither γ-power [data not shown; ANOVA γ-power: frequency F (5,137) = 0.801, P = 0.55; flow rate F (1,137) = 0.0001, P = 0.992] nor γ-frequency [ANOVA γ-frequency: frequency F (5,137) = 0.57, P = 0.72; flow rate F (1,137) = 0.7, P = 0.4] was affected by the sampling frequency or flow rate. Only the γ-duration was affected by the sampling frequency; the duration of γ-bursts was significantly longer at 1 Hz than at basal frequency, 4 Hz, or 8 Hz [ANOVA γ-duration: frequency F (5,137) = 3.970, P < 0.01; flow rate F (1,137) = 0.477, P = 0.49, post hoc Student-Newman-Keuls test].

Finally, the occurrence of LFP γ-oscillations decreased when the sampling frequency increased, and a higher flow rate compensated for this effect to some extent. To gain further insight into the influence of sampling variations, we next looked at the OB unitary level.

UNITS IN THE ANESTHETIZED EXPERIMENTAL PREPARATION: SAMPLING-RELATED PATTERNS OF MT CELLS PERSIST AT HIGH SAMPLING FREQUENCIES. In total, 18 M/T cells were recorded, with 29 neuron/odor pairs recorded from 10 rats. We measured the percentage of responding cells at each frequency and flow rate combination. Regardless of sampling variation, the responsive-
ness of M/T cells to odor was stable and ranged from 69% to 84% (data not shown). We observed a slight increase in cell responsiveness to odor when the flow rate was increased. It has been shown by others that M/T units in behaving rodents tend to lose their respiratory-patterned discharges during rapid sniffing (Bhalla and Bower 1997; Kay and Laurent 1999; Pager 1985; Rinberg et al. 2006a). Surprisingly, we observed that M/T cells still manifested a sampling-related pattern at high sampling frequencies (Fig. 3B), which confirms the recent results obtained by Carey and Wachowiak (2011). For example, under the 1-Hz and 1,000-ml/min conditions, we observed that 52.4% of cells exhibited sampling-related activity. By plotting M/T cell-spike discharges relative to the animal’s respiratory cycle, we found that the rhythmicity of M/T cells was effectively linked to the imposed sampling and not to the animal’s own respiration (data not shown). In addition to the strong persistence of the sampling-related rhythmicity of M/T cells, we observed that increased sampling frequency led to a progressive decrease in the percentage of cells exhibiting a SYNCHRO pattern from 83.3% in the basal condition to 33.3% in the 10-Hz condition (at 500 ml/min; Fig. 3B). Conversely, an increase in the flow rate (1,000 ml/min) improved these percentages \( \chi^2(1) = 6.786, P < 0.01 \). Indeed, at a high flow rate, >50% of the SYNCHRO patterns persisted, regardless of the sampling frequency (Fig. 3B). Moreover, there was no significant difference between the basal/500-ml/min and the 6-Hz/1,000-ml/min combinations or between the 4-Hz/500-ml/min and 8-Hz/1,000-ml/min combinations. Thus a higher flow rate partially counterbalanced the influence of a high sampling frequency.

We then quantified the phase of the M/T cell discharge according to the sampling cycle, which revealed a cell discharge phase-shift when the sampling frequency increased (Fig. 3A, all cells; Fig. 3C, single cell). This phase-shift was observed in all cells whose SYNCHRO activity persisted over a wide range of frequencies. The phase of the spikes relative to the imposed sampling cycle shifted later in the sampling cycle when the frequency increased. This phase-shift was also observed relative to the LFP sampling-related modulation (data not shown).

To summarize, when the sampling frequency increased, the probability of SYNCHRO patterns decreased, and this tendency could be counterbalanced by a higher flow rate. The sampling frequency also affected the phase of spike discharge relative to the imposed sampling cycle. Next, we asked whether the sampling variation also affected M/T cell response latency.

Effects of sampling frequency and flow rate on the latency of bulbar responses: increased flow rate but not increased sampling frequency decreases the M/T cell response latency. Wesson et al. (2009) showed that the olfactory receptor neurons onset latencies from high frequency sniffing trials were significantly shorter than those from low frequency trials. Can a shortening of latencies be similarly observed in the M/T cell response? To address this question, we needed to analyze an event with a precise latency. We thus focused our analysis on the latency of the first spike, which is a discrete event. Numerous authors have highlighted the importance of spike latency in odor coding (Cury and Uchida 2010; Junek et al. 2010). For this purpose, we measured the latency of the first spike relative to the beginning of the first inspiration after odor onset, as described by others (Cury and Uchida 2010; Wesson et al. 2009) (Fig. 4A). Because we were interested in a short-lived event, we restricted the analysis window to 1 s, as the minimum sampling frequency was 1 Hz. Surprisingly, we did not detect a significant effect of sampling frequency on first spike latency [Fig.
In contrast, an increase in the flow rate led to a significant shortening of first spike latency [Fig. 4, B1 and B2; ANOVA, \( n = 229 \), F (5,217) = 0.671, \( P = 0.64 \)]. In contrast, an increase in the flow rate led to a significant shortening of first spike latency [Fig. 4, B1 and B2; ANOVA, \( n = 229 \), F (5,217) = 0.671, \( P = 0.64 \)]. This result indicates that a higher flow rate, but not a higher sampling frequency, decreased the latency of the bulbar response.

Fig. 3. Sampling patterns persist at high sampling frequencies. A: representation of the activities of the whole population of mitral/tufted (M/T) cells relative to the imposed sampling cycle. Dots represent 1/interspike interval of all units relative to their sampling phase. The sampling cycle is depicted between 0 and 1, where 0 represents the beginning of inspiration, and 0.5 represents the transition between inspiration/expiration (red bar). The pink line represents the 50th percentile. B: percentage of sampling-related M/T cell patterns as a function of the imposed sampling frequency and flow rate (yellow, 1 Hz; orange, basal frequency; red, 4 Hz; violet, 6 Hz; blue, 8 Hz; black, 10 Hz). Number of neuron/odor pairs at 500 ml/min: 20, 18, 21, 23, 18, and 18 for 1 Hz, basal frequency, 4 Hz, 6 Hz, 8 Hz, and 10 Hz, respectively. Number of neuron/odor pairs at 1,000 ml/min: 20, 20, 19, 21, and 21 for 1 Hz, basal, 4 Hz, 6 Hz, 8 Hz, and 10 Hz, respectively. Statistical test: \( \chi^2 \), §§global effect of flow rate (\( P < 0.01 \)). Effect of frequency under 500 ml/min flow-rate condition: 10 Hz vs. Basal (***\( P < 0.01 \)), 10 Hz vs. 4 Hz (**\( P < 0.05 \)). Under 1,000 ml/min condition: 10 Hz vs. Basal (**\( P < 0.01 \)), 10 Hz vs. 4 Hz (**\( P < 0.05 \)). SYNCHRO, Spiking discharge presented synchronized activity relative to the imposed sampling cycle. C: example of the cellular activity occurring during odor presentation showing a phase-shift of the spike discharge as a function of the sampling frequency. Flow rate: 500 ml/min for all frequencies. The circular diagram depicts a sampling cycle. The M/T cell spike discharge is presented relative to the sampling cycle. Examples of M/T cell discharges at 500 ml/min under the 6 sampling frequencies. Colors/frequencies are the same as in B. The dotted lines represent the total discharge of the cell under each sampling frequency. The solid lines represent the mean direction of the M/T cell spike discharge relative to the circular diagram.

Fig. 4. Sampling stronger but not sampling faster decreases the olfactory bulb (OB) response. A: first spike latency is defined as the first spike following the first inspiration after odor onset. B1: mean (SE) first spike latencies are presented as a function of sampling frequency and flow rate (gray, 500 ml/min; black, 1,000 ml/min). Statistical test: factorial ANOVA, §§§\( P < 0.01 \). B2: mean first spike latency (all frequencies averaged) for flow rates of 500 ml/min (gray) and 1,000 ml/min (black); ANOVA, §§\( P < 0.01 \).
To determine the joint effects of sniffing frequency and flow rate, we next looked at how these two parameters evolve in behaving animals and how they change relative to one another.

Experiment 2: Relationship Between Sniffing Frequency and Flow Rate in Behaving Animals

To precisely analyze the sniffing behavior of freely moving rats, we used a whole-body plethysmograph. Analyses were performed on six rats. Sniffing behavior was well described by Welker (1964) and then by Youngentob et al. (1987). Our aim here was not to describe the different sniffing characteristics, as Walker et al. (1997) did previously; rather, our goal was to analyze the relationship between the frequency and flow rate.

First, as described by others (Cury and Uchida 2010; Wesson et al. 2008a), we observed that the distribution of sniffing frequencies was bimodal (Fig. 5, A and B) and reflected the prevalence of two respiration modes: a low frequency mode (1–3 Hz) and a high frequency mode (6–10 Hz). To determine the prevalence of two respiration modes: a low frequency mode (0.564, P < 0.0001, n = 41,717) and a high frequency mode (6–10 Hz). The results are presented in Fig. 5, A and B. To determine whether the frequencies and flow rates covaried, we looked for correlations between the instantaneous sniffing frequency and the peak flow rates during both expiration and inspiration phases. We observed that the sniffing frequency and peak flow rate varied in parallel. Indeed, the correlation between the sniffing frequency and inspiration peak flow rate was highly significant (r = 0.515, P < 0.0001, n = 41,717). Similarly, the correlation between the sniffing frequency and inspiration peak flow rate was highly significant (r = 0.446, P < 0.0001, n = 41,717). The same observations were obtained when the durations of inspiration and expiration were analyzed as a function of peak flow rate. As the duration decreased (i.e., sniffing frequency increased), the peak flow rate increased (for the expiration phase, r = −0.461, P < 0.0001; for the inspiration phase, r = −0.564, P < 0.0001, n = 41,717; the inspiration and expiration durations were examined independently). Thus the sniffing frequency and flow rate can covary in freely moving animals. To compare these data with our electrophysiological data, we merged all of the individual frequencies into six classes corresponding to the six sampling frequencies imposed in our anesthetized preparation (≥0.1 Hz): 1 Hz, 2 Hz, 4 Hz, 6 Hz, 8 Hz, and 10 Hz. The results are presented in Fig. 5, C1 and C2. The data confirm that an increase in sniffing frequency was associated with a significant increase in both expiratory peak flow rate [Fig. 5C1; ANOVA, F (5,4,051) = 191.974, P < 0.0001; post hoc Student-Newman-Keuls test, P < 0.05] and inspiratory flow rate [Fig. 5C2; ANOVA, F (5,4,051) = 314.645, P < 0.0001]. The inspiratory peak flow rate was different between each sniffing frequency, except between 6 Hz and 8 Hz (post hoc Student-Newman-Keuls test, P < 0.05). Volume is an important element affected by sniffing flow rate and frequency. We asked whether an increase in flow rate could maintain constancy for volume. As shown in Fig. 5, D1 and D2, as the sniffing frequency increased, the volume significantly decreased during both the expiration [Fig. 5D1; ANOVA, F (5,4,051) = 273.75, P < 0.0001; post hoc Student-Newman-Keuls test, P < 0.05] and the inspiration [Fig. 5D2; ANOVA, F (5,4,051) = 311.843, P < 0.0001] phase. Overall, our results show that when the sniffing frequency increases, there is a concomitant increase in flow rate in the behaving animal. This tradeoff does not allow the sniffing volume to remain constant. We finally asked whether the persistence of LFP sampling-related modulation could be an effect of anesthesia resulting from the imbalance between the peripheral and central influences.

Experiment 3: LFP Sampling-Related Modulation Persists at a High Sampling Frequency in the Awake

We recorded LFP in the OB and sniffing activity in two awake rats (Fig. 6A). LFP activity in the OB of awake rats has been well described by numerous authors (Freeman 1978; Kay 2005; Martin et al. 2004). Although these authors described a theta rhythm (4–12 Hz) in the OB, none concomitantly recorded the sniffing behavior. We observed that sampling-related modulation was still present in the LFP signal regardless of the animal’s sampling frequency (Fig. 6, B1 and B2). LFP sampling-related modulation and animal respiration were tightly related, and the TFRs of both signals were superimposable (Fig. 6, B1 and B2). Interestingly, abrupt variations in the sniffing frequency were strictly reflected by the LFP activity. Therefore, even in a preparation where central control was not depressed, the LFP signals accurately followed the sniffing modulation up to frequencies as high as 10 Hz, as shown in Fig. 6B2.

Overall, the results show that LFP sampling-related modulation persists at high sampling frequencies in anesthetized and awake rats.

DISCUSSION

Sampling is not only a simple vector for odor molecules but also plays a role in olfactory processing (Mainland and Sobel 2006). Sampling can vary in frequency and flow rate (Youngentob et al. 1987), and the question of how sampling variations affect the activity of the olfactory system is a subject of growing interest. In this study, we asked two major questions: what are the effects of high sampling frequency on OB activity at the levels of M/T cell and network activities? Can sampling flow rate compensate for these effects? We observed a tradeoff effect between sampling frequency and flow rate on some bulbar activity features. This tradeoff allows OB activity to maintain a sampling-related rhythmicity and induces faster odor-evoked responses, and it is used effectively in awake animals, in which we observed that frequency and flow rate often co-increase. To our knowledge, our study is the first to report that the sampling frequency and flow rate can act either independently or synergistically on bulbar output to shape the neuronal message.

Sampling-Related Activity is Maintained at High Sampling Frequencies

We observed that OB sampling-related modulation persisted at high sampling frequencies, although it was weakened (Figs. 1–3). This finding is in agreement with previous studies on olfactory epithelium (Ezeh et al. 1995; Ghatpande and Reisert 2011), glomerular (Spors et al. 2006), and M/T cell activity (Bhalla and Bower 1997; Carey and Wachowiak 2011). The effects of high sampling frequency are probably the result of a decrease in the duration of inspiration, which in turn, results in increased activation of the olfactory epithelium. If this is true, then we can hypothesize that an increased flow rate would compensate for this effect. We observed that increased flow rate compensated, to some extent, for the effects of high sampling frequency by enhancing LFP sampling-related mod-
The only partial compensation of flow rate for the effects of increased frequency is probably because of the loss of volume at higher sniffing frequency (Fig. 1A). Regardless, the increased flow rate allows the OB activity to maintain a sampling-related rhythmicity at high sampling frequencies. This finding was obtained in both the anesthetized and awake preparation (Figs. 5 and 6), and this persistence is likely to be important in terms of inter-area communication (for review, see Kepecs et al. 2006). Indeed, the sampling rhythm is in the...
θ-band (4–12 Hz), and θ-rhythms have been described in systems closely related to or involved in olfactory processing, such as the whiskers (Sobolewski et al. 2011) and the limbic system (Bland 1986; Buzsáki 2002; Komisaruk 1970; Macrides et al. 1982; Vanderwolf 1969). Moreover, the coherence between OB and hippocampal θ-oscillations has been found to be significant only during odor sampling (Kay 2005). Interestingly, the cerebellum, which is activated by sniffing (Sobel et al. 1991), has been shown to express θ-rhythms (Wikgren et al. 2010). θ-Rhythms could thus permit the transmission of olfactory information among the olfactory, limbic, and cerebellar structures and could thus be the basis for the rapid feedback of the olfactory system on sampling control (Johnson et al. 2003). In this view, the maintenance of a sampling-related rhythm represents a key mechanism.

Increased Sampling Flow Rate But Not Increased Sampling Frequency Shortens the OB Response Latency

Multiple psychophysical studies have noted the speed of olfactory discrimination in rodents (Abraham et al. 2004; Rinberg et al. 2006b; Uchida and Mainen 2003), which indicates rapid olfactory processing. We could think that a high sampling frequency would reduce the latency of the bulbar response. Although Wesson et al. (2009) showed that this holds true for neuroreceptor responses in awake animals, we observed that a high sampling frequency did not significantly impact the latency of the M/T cell response. Our observation is in agreement with the studies by Carey and Wachowiak (2011) and Spors et al. (2006). More interestingly, we observed that the M/T cell response latency can be reduced by increasing the flow rate. Whereas other authors have shown that increasing odor concentration decreases the M/T cell-firing latency (Cang and Isaacson 2003; Wellis et al. 1989), this is the first evidence to show that a sampling parameter alone (i.e., flow rate) can impact the M/T cell response latency. This result may reconcile the data of Wesson et al. (2009) with ours, because they did not specify whether a high sampling frequency was associated with an increase in flow rate in their preparation. Because discrimination can be achieved in <500 ms (Abraham et al. 2004; Rinberg et al. 2006b; Uchida and Mainen 2003), the olfactory system must operate on a rapid timescale to provide a quick response. We demonstrated that it is possible to shorten the OB response latency by increasing the sampling flow rate.

We observed a minimal and constant OB response latency regardless of the sampling frequency. Multiple arguments, which are not mutually exclusive, can be proposed to explain this latency. First, there is an incompressible time period that corresponds to information transduction in the olfactory epithelium and/or conduction to the OB (Grosmaire et al. 2006; Kleene 2008). Second, the shortness of inspiration at a high sampling frequency might need to be compensated for by the cumulative effect of several cycles of olfactory epithelium activation. Furthermore, this constant latency is probably related to the observed phase-shift in the synchronized pattern relative to the imposed sampling cycle. Because odor onset and odor response latency were fixed, whereas the sampling frequency increased, the first M/T cell spike was shifted relative to the imposed sampling cycle. This is a possible explanation, and it does not exclude the alternatives, such as a differential odorant deposition, as a function of sampling frequency (Jiang and Zhao 2010). This observed shift could also be the result of a modulation of granular inhibition induced by stimulus frequency (Young and Wilson 1999) or excitatory centrifugal input (Balu et al. 2007). A phase-shift of M/T cell discharge could have important consequences for downstream area reading, for example, by changing the respiratory phase of piriform cortex cells (Litaudon et al. 2003; Wilson 1998). The phase-shift of the M/T cell discharge could encode a stimulus parameter, such as a change in sampling frequency or a change in stimulus concentration because of the reduced inspiration time (Kepecs et al. 2006).

Different Sniffing Strategies Right Under The Nose

The last part of our results (Fig. 5) shows a co-increase between the sampling frequency and flow rate with a correlation of up to 0.5. This result also means that other strategies could exist, such as combinations of low flow rate/high sampling frequency or high flow rate/low frequency. Moreover, previous studies have shown that sniffing varies depending on the task (Kepecs et al. 2007; Wesson et al. 2008b; Youngentob et al. 1987). Thus an animal has the ability to combine various sniffing parameters differently according to the task and/or environment, which likely confers an important degree of adaptability to the olfactory system. As...
suggested by Schoenfeld and Cleland (2005, 2006), sampling can improve olfactory capabilities by allowing the optimization of the deposition of odor molecules through the olfactory epithelium. Further studies using behaving animals will be required to gain insight into how the olfactory system can control sniffing and to provide evidence for an olfactory motor act.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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